

## GENETIC AND DEVELOPMENTAL STUDIES ON *BOTRYLLUS SCHLOSSERI*<sup>1</sup>

ROGER MILKMAN

*Marine Biological Laboratory, Woods Hole, Massachusetts 02543 and Department of Zoology,  
Syracuse University, Syracuse, New York 13210*

The colorful compound ascidian, *Botryllus schlosseri*, has great promise for investigation in several important theaters of genetics, notably development and natural variation. In order for its potential to be realized as an experimental animal in these areas, a variety of preliminary studies have been undertaken. The results of these studies are reported here.

*Botryllus* has been studied extensively by Bancroft (1903), Berrill (1941a, 1941b, 1951, 1961), Oka and Watanabe (1957, 1959, 1960), and Sabbadin (1958, 1959, 1960, 1962, 1964), as well as by Scott (1934), Watterson (1945), and others. After Bancroft's early work on its natural history, development, species structure, and its property of colony fusion, Berrill used *Botryllus* as one major object of study in his broad and highly important series of contributions on development. More recently, Oka and Watanabe (on *Botryllus primigenus* and *Botrylloides*) and Sabbadin have addressed themselves to additional developmental problems, as well as to the genetic analysis of pigmentation and compatibility.

Although *Botryllus* is well described in the literature (Berrill, 1950; Van Name, 1945) and is exceedingly common just below the low water mark on pilings, eel grass, and under rocks, particularly in harbors, it is not a familiar organism even to many marine biologists, and a brief description is therefore in order. *Botryllus* colonies are of irregular shape and may be well over a foot in diameter, though usually much smaller. Each colony (Fig. 1) is composed of rosette-like systems of generally 5–18 zooids, each of which is like a solitary ascidian in form. The zooids, together with a vascular system which pervades them and the areas between and around systems, and which consists of blood vessels and ampullae (Fig. 2), are embedded in a gelatinous matrix which is maintained in a dynamic state by the activities of numerous amoeboid cells. The zooids' long axes are radially arranged in the systems. Their oral (incurrent) siphons are peripheral and open directly to the water; their atrial siphons open into the system's common atrial chamber which in turn communicates with the outside via a common atrial opening. The concentration of hydraulic force thus permits the powerful ejection of fecal pellets (and sperm); accordingly the system may be thought of primarily as a unit of egestion.

The oozoid resulting from the metamorphosis of a tadpole-type larva initiates the asexual formation of a colony by budding. Throughout the life of the colony, budding is synchronous, and when the buds become functional zooids, the previous

<sup>1</sup> This was supported by Research Grant GM 07810 of the National Institute of General Medical Sciences, United States Public Health Service.

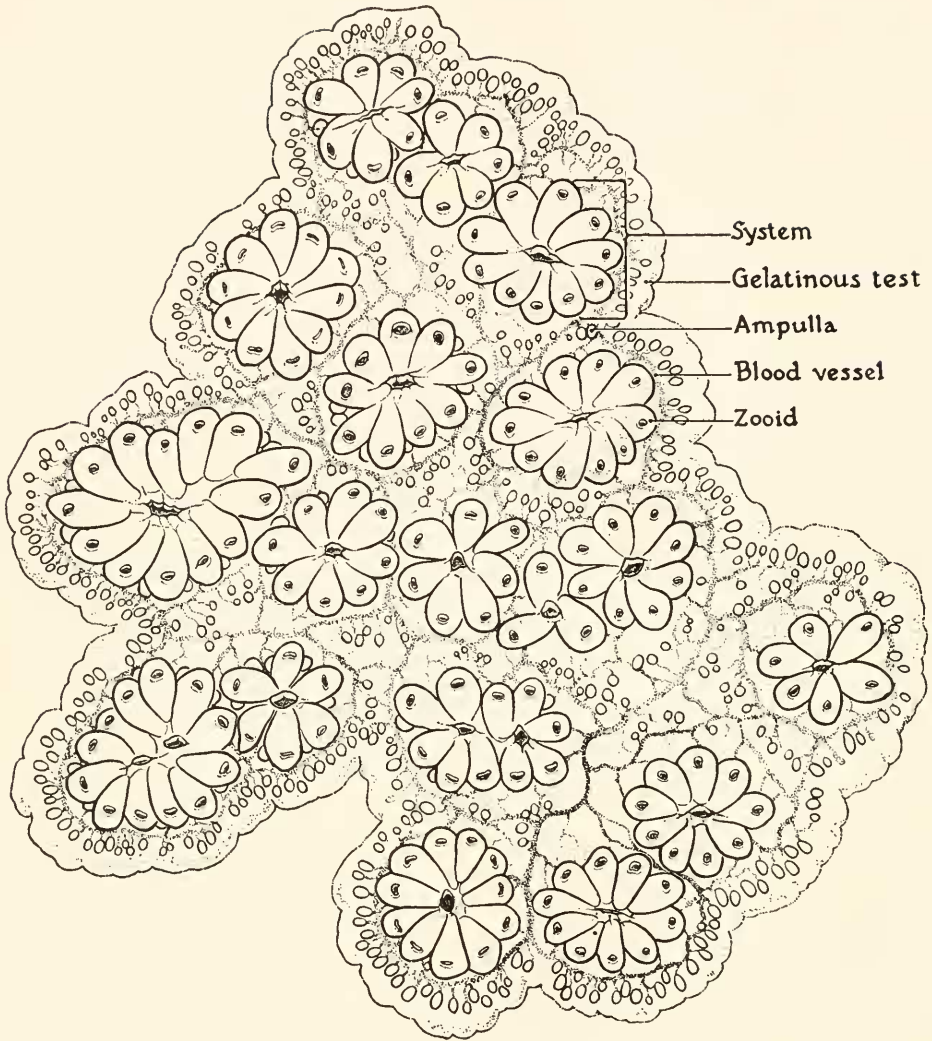


FIGURE 1. Habit sketch of *B. schlosseri* on glass.

generation of zooids is resorbed. Beginning with the oozoid, zooid size and pigmentation increase in each of the first five or ten asexual generations. During this period, first functional sperm and finally mature eggs make their appearance. The buds are produced at specific sites on the atrial wall, one per zooid at first, and later up to four. The dependence of functional gonads upon a certain zooid size suggested, and specific surgical experiments (Berrill, 1961) confirmed, that the degree of differentiation is dependent on mass in a manner reminiscent of the findings of Lopaschov (1935) and Grobstein and Zwilling (1953) in frog, chick, and mouse. Colonies under suboptimal conditions may mark time or even regress while the sequence of budding and resorption continues.

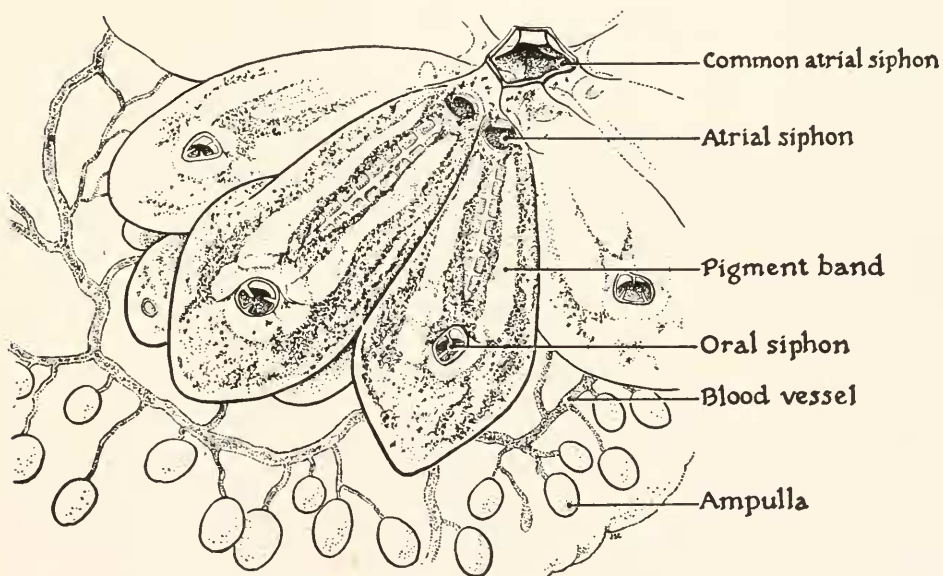
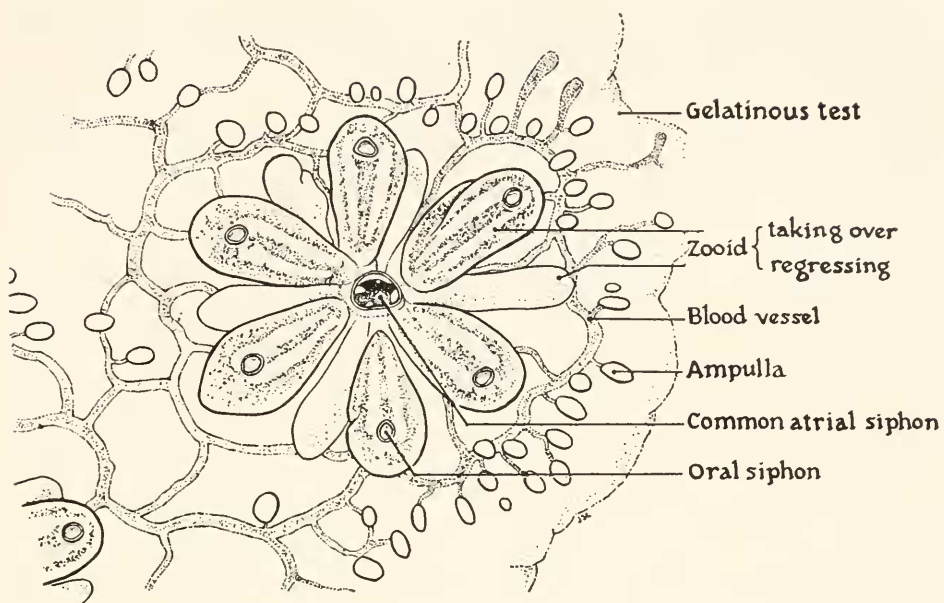


FIGURE 2. Details of colony in Figure 1.

Sabbadin (1958) has also caused right-left inversion of asymmetry by disturbing early buds. This *situs inversus* is perpetuated faithfully in further atrially budded generations. We have repeated these observations.

Since all the zooids in a colony are ordinarily derived by budding from a single progenitor, the colony is a clone, and all the individuals share a color pattern which is distinguished easily from most of the myriad color patterns of surrounding colonies. Age and environmental influences on color patterns exist (Watterson, 1945) but are subject to independent analysis. Contiguous colonies are delineated by a clear discontinuity, generally bordered by tiers of vascular ampullae. Since the colonies occasionally fuse (the possibility apparently being based on genetically controlled affinities), mosaics do arise. These mosaics may become quite complex, since each generation of zooids is resorbed when its buds mature, and the systems can be rearranged radically—indeed, shuffled—as the number of zooids changes. The basis of the color pattern lies in the number and distribution of three kinds of pigment cell: blue (granular), white (granular—purine derivative), and orange (carotenoid in solution) (Sabbadin, 1959).

Experiments by Sabbadin (1959, 1962, 1964) show that certain components of color patterns are inherited in a simple Mendelian way. The availability of a large number of potential markers, together with other useful properties, suggests the feasibility of extensive genetic studies on *Botryllus*. This accessibility to classic genetic analysis is fortunate in view of the major modern problems for whose investigations it appears remarkably well suited. Sabbadin (1959) has pointed out that the tremendous variety of color patterns, once resolved into the activities of individual genes, would offer a way to study natural genetic variation by direct observation. In addition, of course, subsequent studies of the individuals observed and their progeny would add further substance to such an investigation. The primary concern of the present paper, however, is development. The remarkable powers of regeneration shown by *Botryllus* put it in the same league as many plants, such as carrot and tobacco; moreover, its structural complexity and the similarities of its larval development to that in some vertebrates add to its desirability for study. Finally, it is the hope of many animal geneticists to establish cell culture operations by which they can alternately treat cells as micro-organisms for mutational and recombinational studies, and grow them into adult organisms for detailed study of form and function. Thus an important part of the genetics of development may be accessible to analysis in *Botryllus*. The experiments to be described may be viewed as steps in this direction.

#### LABORATORY CULTURE

*Botryllus* can easily be maintained in laboratory culture, provided that certain conditions are met. Cultures must be flat, rather than in clumps. The flat growth habit is automatic when larvae settle on glass slides or similar flat objects and metamorphose. Flat colonies from large mussel shells, boards, or similar natural surfaces can easily be removed and allowed to attach to glass; small clumps, if attached to a flat surface, will also spread out by the movement (on the order of several mm./day) of the existing systems and with subsequent growth.

The physical circumstances of the colonies in culture are critical. Beakers of still sea water serve well. Larvae will attach to glass slides, which can then be

placed vertically, or even better, aslant with the *Botryllus* facing down. Horizontal mounting, with the colonies upside down, is best of all: the fecal pellets drop away, and contaminating filamentous algae are less likely to take hold. Saran wrap, to which the larvae readily attach, can be floated on culture medium also. For examination, it is inverted and submerged; it can be refloated when desirable. Also, for fusion compatibility tests, the Saran can be cut and colonies approximated, with a firm supporting substratum if desired. Zooids on the bottom of a vessel simply do not do very well.

Turbulence results in the presence of fecal pellets and other comparably sized detritus throughout the sea water: contact of such particles with the oral siphon or branchial basket causes reflex cessation of pumping and reversal of water flow by contraction of the body wall and thus prevents feeding and a normal flow of water through the individuals. If such a situation persists, the colony degenerates. Accordingly, aeration and stirring, if employed, require careful design.

*Botryllus* is tolerant of salinity changes. Concentration or dilution of sea water by 20% produces no ill effects, and short exposures to more extreme conditions (including distilled water) can be survived. The use of Instant Ocean, an artificial sea water, is advantageous from several points of view. It contains no organic substances and no predators or competitors, and it is a great convenience inland. Under conditions where evaporation is controlled or compensated and micro-organisms do not multiply explosively, weekly changes of water suffice.

Cultures grow well between 18° and 28° C. Since water temperatures survived over the winter are much lower, it is likely that lower culture temperatures could be used, but growth would be very slow at best. It is also probable that even higher temperatures could be used, particularly where other conditions are optimal.

Cultures can sometimes survive for months without added food. The budding cycle proceeds slowly with a gradual reduction in size and number of individual zooids. This suggests that rapid regression and death are not due to starvation, and thus that a major problem in laboratory culture is the control of other organisms. Colony growth, of course, requires the addition of food (algae), either *via* running sea water or from algal cultures. *Cyclotella nana*, a centric diatom, appears to be the best food organism used so far. In f/2 medium (Guillard and Ryther, 1962) made with Instant Ocean the algal cultures reach concentrations of  $1 \times 10^6$  cells/ml.; in f/2 medium made from sea water,  $2 \times 10^6$  cells/ml. *Botryllus* colonies grow well in concentrations of  $0.5\text{--}2.5 \times 10^5$  cells/ml. Indeed, young zooids under these conditions have on occasion developed four buds each, one more than the three considered maximal till now (Berrill, 1961), and the four buds have all become functional zooids in some cases.

Satisfactory feeding of any filter-feeder requires that two conditions be met: first, there must be enough food, and second, the concentration of this food must be high enough for an adequate feeding rate but not high enough to be harmful. In the present case, a concentration of  $1\text{--}2 \times 10^5$  cells/ml. is used. This is a safe distance from the level at which the feeding system becomes clogged, too many algae accumulate on the dorsal lamina, periodic regurgitation takes place, and death eventually ensues. *Botryllus* is apparently not successful at intermittent feeding in constant high concentrations of food. A concentration of  $5 \times 10^5$  cells/ml., for example, is accompanied by slow growth and poor appearance of the colonies.

Higher concentrations generally cause regression after a day or so. On the other hand,  $0.5 \times 10^5$  (a concentration which also supports efficient feeding) has the disadvantage of providing only  $\frac{1}{4}$  as many diatoms as  $2 \times 10^5$  in a given vessel, thus necessitating a volume four times as great.

With just a few newly metamorphosed oozoids, culture vessel volume is no problem; but with colony growth, the removal of algae from the medium becomes rapid. On the basis of the time taken for a given colony to clear its water, I estimate that each zooid can easily filter  $2 \times 10^6$  algae per day. (Not all of these are absorbed, as examination of the fecal pellets shows, but they are no longer available.) Thus, if a suspension of  $2 \times 10^5$  algae/ml. is provided each day, the minimum culture vessel volume is about 10 ml. for each zooid. Since food intake becomes slower as the concentration falls, doubling this volume would be even better. A suspension of  $1 \times 10^5$  algae/ml., allowing 40 ml./zooid, is probably optimal.

Closed vessels (or vessels covered with glass plates, Parafilm, *etc.*) are obviously convenient, particularly since stirring is not required. Polyethylene, through which oxygen and carbon dioxide can pass and water vapor cannot, suggests itself as a good cover (Walters and Williams, 1966). Constant light is acceptable and permits the diatoms to photosynthesize and thus produce oxygen. It also favors the growth of all algae, however, and may do more harm than good if certain filamentous forms are present. Budding and gonad development proceed similarly on all light regimes; only larval release (Costello *et al.*, 1957) seems to be influenced by light. In any event, large colonies require impractical volumes in standing culture, so continuous flow systems maintaining the concentration of algae within the desired range are preferable for them. The development of a recirculating aquarium for filter-feeders would be useful: the problem is merely one of finding an appropriate water filter.

For genetic studies, rapid growth and sexual maturation are desirable. Under the culture conditions described, performance is satisfactory. In the progeny of one mating, the colonies had from 5–22 zooids 22 days after metamorphosis (26 days after fertilization). Mature eggs are produced by cultured colonies less than  $1\frac{1}{2}$  months after metamorphosis; and once eggs are produced, of course, a new hatch appears every 5–7 days, as long as conditions remain good.

Sabbadin (1960) has reported using *Chlamydomonas* (marine members of this genus are now called *Dunaliella*) and *Nitzschia* (perhaps what is now called *Phacodactylum*) to feed isolated colonies at Chioggia, on the Lagoon of Venice. I am not convinced that I have given this combination an adequate test, but I have not been successful with it.

Predators, such as the snail *Mitrella lunata* and probably some flatworms and nematodes; competitors, such as filamentous algae, sponges, encrusting ectoprocts, *Bugula*, and entoprocts; and bacteria (whose activities may be competitive or direct) can all destroy cultures. In running sea water, *Mitrella*, sponges, and ectoprocts become an increasing problem as the summer progresses. In isolated culture, bacteria, algae, and entoprocts have proven more bothersome. Amphipods, harpacticoid copepods, and a variety of ciliates seem to coexist peacefully in *Botryllus* cultures as they do in the miniature jungles of wild colonies. But clean colonies derived from washed unhatched larvae do best.

Although in the long run *Botryllus* cultures require the maintenance of favorable conditions, they respond well to occasional rough treatment. For example, small colonies on glass have survived exposure to air for ten minutes or longer and microscopic examination under a coverslip for similar periods. Colonies accustomed to 25° C. have survived a day in the refrigerator, but not much longer.

### THE REPRODUCTIVE CYCLE

It is of particular importance when dealing with an organism capable of selfing to have control of fertilization. Such control is achieved in *B. schlosseri* by fertilizing isolated eggs with isolated sperm (Milkman and Borgmann, 1963). It is believed that this is the first time external fertilization has been accomplished with a compound ascidian, and it depends upon removing the eggs at the right time. This in turn depends upon a detailed understanding of the timing of egg maturation and sperm maturation in relation to one another and to the asexual cycle. The present investigation has clarified these time relationships.

TABLE I  
*Timetable for one asexual generation*

Day	Adult	Embryo	Testes	Bud	Egg	Testes
0	Takes over	Fertilized	0-1	Small	Small	
1	Darkens	Raspberry	1-2	Grows and		
2	Grows very	Tailbud	2-3	projects out		
3	little	Wraparound	2-4	between zooids		
4		Larva	3-5			
5		Released	4-remnants	Swells and	Full-	pre-0
6	Resorbed			takes over	sized*	pre-0-0 0-1

\* Enters atrial cavity, germinal vesicle breaks downs, egg fertilized.

Eggs develop in special chambers beside growing buds. They reach maturity when the buds replace the previous zooid generation. Since this is a fairly synchronous process (distant systems in a large colony may be several hours apart), one can obtain hundreds, even thousands, of eggs from a good-sized colony. As the new generation takes over, the eggs are pushed out of their chambers into the atrial cavity of the swelling bud. The germinal vesicle is in clear evidence in the eggs. During the next two to three hours, the following things happen in parallel: (1) the old zooids shrink down and no longer contain (or release) sperm; (2) the new zooids swell further and open their siphons; (3) the germinal vesicle breaks down and the eggs soon become fertilizable.

It should be added that the new zooid's testes generally do not release mature sperm until two days later. From this array of events, then, it follows that eggs will not be fertilized by sperm from the same colony-clone unless sperm are not forthcoming from elsewhere for two days, or unless the colony is so large that the first eggs become accessible and fertilizable before the last old zooids, often virile to the end, lose their mature sperm.

At the time of takeover, the testes contain very few mature sperm. That these are not released is indicated by the failure of newly mature eggs in the same colony

or another to be fertilized by them. The proportion of mature sperm in a testis rises with time. The rate of release must be low at first; the testes ultimately reach a state of great fragility in which they contain nothing cellular except sperm oriented in parallel; at this point the sperm output of the colony must be several orders of magnitude greater than at first. Table I shows the timetable of sexual and asexual events in an adult colony. The budding cycle takes 5-7 days in the laboratory. Illustrated in Table I is a 6-day cycle. The embryonic stages' designations used here for convenience, reflect their appearance. The raspberry stage is a gastrula; the wraparound is still rather opaque and spherical, with the tail wrapped around. Later the embryo clears and elongates into the larva, whose subsequent release appears to be influenced by its light regime (Costello *et al.*, 1957). The buds are not visible until just before takeover, except in very flat colonies. Otherwise they are concealed in the interior of the colony mass.

TABLE II  
*Distribution of testis stages vs. embryo stage in individual colonies*

Colony	Embryo stage	Testes in each stage (N)					
		0	1	2	3	4	5
1	Raspberry	0	1	22	2	0	0
2	Raspberry	0	4	23	3	0	0
3	Tailbud	0	2	22	1	0	0
4	Tailbud	0	0	9	11	1	0
5	Early wraparound	0	1	13	2	0	0
6	Wraparound	0	0	1	22	0	0
7	Larva	2*	1*	1	18	0	0
8	Larva	0	0	0	3	17	1
9	Tadpole	1*	1*	0	0	0	15
10	Tadpole rare	0	2	0	0	0	20

\* Possibly taken accidentally from buds (see text).

Table II shows the degree of uniformity among the testes in a colony. Colonies were staged according to their embryos, whose stages are very uniform indeed under normal circumstances. Since these colonies were taken from a dock crowded with *Botryllus*, their eggs were surely fertilized at the earliest possible moment. Now within a given colony, the testes appear to be fairly synchronized, though there is some scatter. (Exceptionally immature testes in an otherwise mature group may have been taken accidentally from a bud.) On the other hand, a comparison from colony to colony suggests that the phase relationship is not constant for the species, though of course its range of variation is not great. This variability among colonies must be kept in mind, for it, too, affects the possibility and time of selfing.

It should be clear from this description that there is no sudden onset of paternal competence in a *B. schlosseri* colony. Mature sperm are seen well before they are normally released; crushed testes achieve a small percentage of fertilization at early stages also. Table III illustrates the quantitative nature of testis maturity, comparing testis stage with per cent fertilization. Eggs from the same batch were placed with crushed testes of various stages and cleavage was observed.

Conjectures involving storage of sperm or other complex mechanisms of fertilization can be discarded because eggs can be taken at the right moment and fertilized. Similarly, there is no evidence of egg-sperm incompatibility. The only technical difficulty is that eggs isolated with germinal vesicles intact will never be fertilized; and it is probable that they do not mature until about an hour after breakdown. Subsequently the eggs can be removed and fertilized. The actual removal consists of slitting the zooids and gently pressing out the eggs: this is a very easy procedure, and the zooids repair the damage within 24 hours.

Large wild colonies containing many eggs per zooid (I have removed as many as 11 from one) can be staged and isolated about a day before takeover. As Sabbadin points out (1959), the property of fusion places the clonal nature of any wild colony in doubt, however, and it is certainly better to raise breeding colonies from tadpoles. At any rate, for eggs each colony should be sequestered before the new siphons open. For large colonies, a lucite container, divided into radial sectors, has been used to isolate up to ten potential egg sources. This device, built to operate like a reverse *Botryllus*, distributes water from a common central tube; outflow is

TABLE III  
*Fertilization efficiency vs. testis stage*

Testis stage	Eggs (N)	% Fertilized
1	55	0
3	135	15
3½	80	28
3½	62	29
4+	83	46

peripheral. Where running sea water is not available, large chambers containing no food can be used. Here stirring involves no risk. A closed vessel, together with the great efficiency of the *Botryllus* pumping system, raises the problem of selfing once more (which is minimized by the constant washing of continuous flow). Accordingly, egg sources should be washed and isolated shortly before the new siphons open.

Eggs to be fertilized *in vitro* are placed in a Syracuse dish of (natural or artificial) sea water. Testes are then added. After the eggs are swirled to the center of the dish, the testes are crushed, and the eggs are nested in a thick layer of sperm like berries in whipped cream. Polyspermy is fortunately not a problem, and fantastic quantities of sperm are required in comparison, for example, to the amounts needed to fertilize sea urchin eggs. This situation, seen somewhat less spectacularly in other tunicates (Costello *et al.*, 1957), suggests that imperfections still remain in the *in vitro* method, even though 100% fertilization can be achieved.

#### DEVELOPMENT OF FERTILIZED EGGS

Eggs fertilized *in vitro*, as well as early embryos removed from zooids, can develop into mature larvae and subsequently metamorphose. Until recently, techniques for permitting such *in vitro* development were complicated and unreliable at best; this was a major obstacle to the use of controlled mating. Now, however, the

simple expedient of placing the early embryos on a piece of filter paper in a vessel containing Instant Ocean provides a good method of raising them. The filter paper apparently serves two purposes: it provides for some circulation even where the egg rests on its surface (Saran doesn't work as well), and it is not as hard as glass. Eggs resting directly on glass become deformed and bacteria accumulate at the point of contact and almost invariably attack and destroy the embryo by the time of the gastrula stage or thereabouts. Filtered sea water is also satisfactory. If evaporation is prevented, the water need not be changed, but a change seems to result in healthier larvae. Hundreds can be raised in a finger bowl. Before eclosion, the larvae are transferred to an appropriate vessel containing slides or Saran for attachment, since the time of eclosion and that of attachment and metamorphosis are variable and hard to control.

#### THE VASCULAR SYSTEM AND VASCULAR BUDDING

The vessels which pervade the zooids and outlying areas also connect to the many vascular ampullae which are found in tiers at the periphery of the colony, in rings around each system, and irregularly scattered throughout the matrix between systems. Differential interference (Zeiss-Nomarski system) microscopy, which permits undistorted high-magnification observation of optical sections of relatively thick preparations, shows that the vessels and ampullae are very delicate structures. Their walls are essentially one cell thick. Ordinary light microscopy of young colonies under coverslips permits similar observations. The blood circulating through the systems is moved, not only by the hearts in the various zooids, but by contractions of the vascular ampullae. This is confirmed by motion pictures, again using the Nomarski microscope, which show localized contractions within each ampulla and thereby eliminate passive elastic contraction as the basis of their periodic reductions in size. Moreover, removal of all the zooids and buds does not stop circulation in the remaining outlying vessels. Circulation continues for hours, and longer.

Isolated regions of the vascular system are of great interest because they can regenerate entire zooids and, ultimately, whole colonies, in spite of their simple structure and composition. Oka and Watanabe (1957, 1959) demonstrated vascular budding in *B. primigenus* and in *Botrylloides*; Watkins (1958) suspected it in *B. schlosseri*; and Byrne and I demonstrated it (Milkman and Byrne, 1961). In *B. schlosseri* vascular budding has been seen only when all the zooids are removed, but the possibility remains that under conditions of very rapid growth it occurs in intact colonies also. During the first few days following excision of the zooids (and buds!), the ampullae consolidate into one or a few rather highly pigmented masses, and these structures may now gradually begin to resemble miniature zooids, whose size is that of zooids newly metamorphosed from larvae. Histological study of this process has not yet been made in *B. schlosseri*, but even at the gross level there appear to be differences between vascular budding in this species and those reported by Oka and Watanabe. In less than a week, the tiny functional zooids are feeding and growing.

This remarkable regenerative ability leads one to wonder if *B. schlosseri* cells can be cultured and then be induced to form zooids in a manner analogous to vascular budding. It was considered useful first to make further inquiry into the nature

of the cells determining the characteristics of the zooids so produced. To this end, advantage was taken of the ability of morphologically different colonies to fuse (Bancroft, 1903). Colonies differing in color pattern were made contiguous; a small proportion of them fused gelatinous tests and vascular systems, thus permitting (indeed, necessitating) a complete interchange of blood cells. A week after fusion, all zooids and buds were removed from the fused colonies, leaving only their common vascular systems (and tests). Separate portions of each colony were taken before fusion and maintained for comparison with the zooids to be regenerated. In over 30 cases, the zooids produced by vascular budding in turn produced systems identical to the parent systems originally present at the site of the bud. There were no exceptions. This proves that, whatever the contributions of the freely circulating blood cells, the fixed cells of the delicate vascular walls (or conceivably of the test) determine the phenotype of the regenerated zooids (Milkman and Therrien, 1965). Figure 3 illustrates the experiment. No buds were seen at the original fusion border; this is probably a statistical matter. Perhaps a somatic recombinant could be obtained if a bud of dual origin occurred.

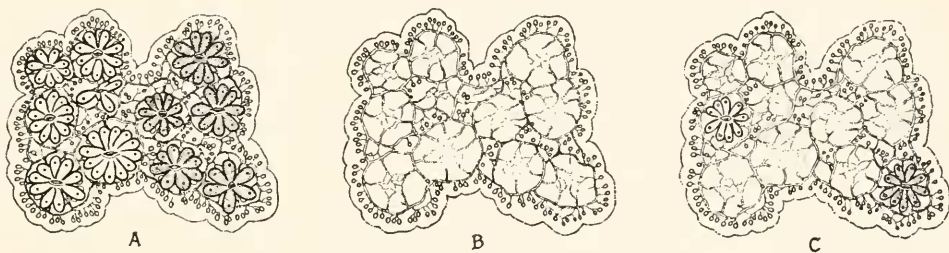


FIGURE 3. Experiment on vascular budding in combination of two fused colonies. Note similarity of system derived from vascular buds to systems derived from vascular buds to systems originally present at the same site. See text for further details.

In these experiments, it was necessary for the systems produced to reach a steady-state with respect to pigment cell concentration. During the first several atriol budding generations after the vascular bud is formed, the proportion of pigment cells is unusually high, as it is in the original coalesced vascular tissue. Gradually this proportion decreases and is maintained at a steady level corresponding to that in the parent. This situation is reminiscent of the changes in pigmentation in the first few bud generations coming from an oozoid; but in the latter case, pigment cell concentration starts low and then rises to a maintained level (Bancroft, 1903; Watterson, 1945; Sabbadin, 1959). Moreover, when the old zooids are resorbed, their pigment cells assemble in the ampullae and are gradually released and taken up by the new generation of zooids. Thus buds begin by being rather pale; their sudden expansion at takeover time spreads out their relatively few pigment cells and makes them paler; and they gradually darken the next day. From these observations, old and new, it can be seen that the distinctive pigmentation of a mature *Botryllus* colony depends basically on its own particular genotype, and that it is affected more immediately during the ontogeny of the colony by the relative rates of formation of pigment cells and their concentration in a given stage (oozoid or regenerating tissue).

The obvious next step in this area of investigation, which has not yet been undertaken, is an attempt at culturing vascular tissue, with or without blood cells. It is of interest that Freeman (1964) has found in *Perophora* that only lymphocytes, of the several varieties of blood cells, are necessary for budding, which occurs normally in that form at intervals at the growing end of the stolon, which carries bloods among the zooids. Since he arrested all cell division by irradiation and subsequently injected untreated cells of a given type, it is clear that these cells form the body of the newly budded zooids, unless (as seems unlikely) large numbers of non-dividing cells are mobilized from existing zooids once the blood cells initiate budding. Our findings do not rule out the participation of blood cells, but they suggest that the structure of the *Botryllus* zooid formed in vascular budding is controlled by the derivatives of the vascular tissue, or conceivably (though the appearance of the bud lends no support to this alternative) cells in the test. The quantitative changes in pigmentation suggest that different regions in the zooids have different affinities for each kind of pigment cell and that a mass action relation-

TABLE IV  
*Results of representative crosses*

Cross	Phenotypes	Genotypes	Phenotypes in offspring	
1 (self)	PB × PB	PP Bb × PP Bb	20 P:0 p	15 B:5 b
2	pB × PB	pp Bb* × PP BB	15 P:0 p	15 B:0 b
3	Pb × PB	P-† bb × P-† Bb*	19 P:0 p	7 B:12 b

Pigment band: P presence, p absence. Black ground color: B presence, b absence.

\* Bb genotype indicated by another cross of same parent (not shown here).

† At least one should be PP.

ship determines the disposition of pigment cells at any time. The mobility of pigment cells that has been observed supports the view that, though they may lodge in a particular place for a considerable time, they are never permanently fixed.

#### GENETIC CROSSES

The crosses we have performed so far have been preliminary in nature and lead to three conclusions. First, selfing is general enough to suggest the absence of any important self-incompatibility system (except, of course, the highly effective difference in time of maturation of eggs and testes in a given colony). Second, Sabbadin's conclusion that the presence of a pigment band may be inherited in a simple, dominant Mendelian fashion is supported, although neither his data from individual crosses (Sabbadin, 1959, 1962, 1964) nor ours definitively exclude additional possibilities. Third, it is clear that the tremendous variety of offspring produced from a cross of any two colonies taken from nature defy extensive analysis: several generations of selfing are required to produce colonies sufficiently homozygous to be useful for the study of a large number of traits. Such a program of selfing necessitates laboratory culture methods capable of supporting sexual reproduction consistently; even now that we have such methods, any major degree of heterosis might delay or prevent the acquisition of homozygous colonies.

Table IV contains the results of some representative crosses. Putative parental genotypes for pigment bands are assigned tentatively; it is not really clear at this point that the inheritance of black ground color is simple. The numbers are quite small, but since these crosses were set up the techniques for getting good yields have been improved greatly.

There is one detrimental result of *in vivo* selfing of the usual type: when self-fertilization takes place two days after the normal time, the larvae are not ready for release at takeover time (see Table I). The colony seems unable to adjust its asexual schedule; the old zooids regress slowly and incompletely while they contain larvae. Concomitantly, the buds do not complete their last stages of development: they appear undersized and do not become contiguous with other zooids to form normal systems. Thus the colony dies, though many larvae escape. In exceptional cases of earlier selfing due to fertilization by remaining zooids of the previous generation or by unusually advanced testes of the current generation, this collapse may not occur. Actually, the fortunate expedient of refrigerating testes up to four days may be employed; sperm from these testes fertilize the eggs of the next generation perfectly. In addition, the possibility also exists of separating a colony into parts and staggering them at different temperatures.

Where traits are inherited in a simple Mendelian fashion, the alleles responsible can be followed in populations. As Sabbadin has suggested (1959), *Botryllus* is of particular interest because in animals two alleles associated with a striking phenotypic difference rarely both have high frequencies. The pigment band's presence appears to be dominant over its absence, although Sabbadin (1964) believes that multiple alleles account for some of the variants in pigment band form. At any rate, among 100 colonies on the M. B. L. Supply Dock in Woods Hole, 63 had pigment bands and 37 did not. If the conditions for the Hardy-Weinberg law obtained, the frequency of the "absence" allele was  $\sqrt{0.37} = 0.6$ , while that of the "presence" allele, or class of alleles, was  $1 - 0.6 = 0.4$ . Extension of these observations over space and time should be quite easy and may lead to useful conclusions about population sizes and related matters.

#### CHROMOSOME NUMBER

Colombera (1963), using either gallocyanin or gentian violet, together with preliminary aceto-orcein or aceto-carmin staining, on testes, buds, and cleaving eggs, has concluded that the haploid number of chromosomes in *B. schlosseri* is 16. Therrien and I (Milkman and Therrien, 1965) studied cleaving eggs using the Feulgen technique, blockading the cytoplasmic aldehyde groups with phenylhydrazine before hydrolysis. (Pronase had removed the chorion.) We estimated the haploid number to be 7 or 8. It is possible that this disparity has a real basis, or, of course, that our conclusions are incorrect. Colombera points out that 16 is rather high for ascidians, but that *Tethyum plicatum*, of the Styelidae (a family close to, or including, the botryllids) also has a haploid number of 16.

#### HANDLING OF BLOOD

In my laboratory, Dr. Arnold Kahn has found it easy to remove and reinject *Botryllus* blood. As much as  $\frac{1}{4}$  ml. of blood has been taken from a colony at one

time, suggesting that the alternate passive elastic stretching and active contraction of ampullae are ordinarily responsible for the periodic reversal of peripheral blood flow. Blood cells and test cells survive in culture for up to three days but do not multiply. The ability to remove and inject blood easily and without injury permits one to attempt to confer fusion compatibility. It is also conceivable that intravenous feeding alone can support the growth of a *Botryllus* colony or isolated vascular system, and that nutritional studies at this level might lead to a wide variety of interesting findings.

#### GENERAL DISCUSSION AND CONCLUSIONS

The basic technical obstacles having been overcome, we can now look forward to extensive genetic studies on *Botryllus*. Inland culture techniques permit year-round propagation of strains, together with long-term crossing and selection programs. The ever-present risk of selfing, which cannot be controlled *in vivo*, is eliminated by the use of *in vivo* matings, which also permit multiple crosses involving one set of eggs or one set of testes. It may be concluded, then, that *Botryllus schlosseri* is ripe for teaching and experimental use. In anticipation of its increased popularity, in view of its appearance and habitat, and to remedy a current defect, the vernacular name "harbor stars" is now suggested.

This work has been done with the collaboration and assistance of Sylvia Byrne and Edward Therrien (NSF Undergraduate Research Participants), Martha Borgmann and Judith Pederson. Dr. Luigi Provasoli, Dr. Robert Guillard, and Mrs. Helen Stanley have been most generous with algal cultures, materials, and counsel. Dr. Martha Baylor suggested the fusion-vascular budding experiment. Dr. Robert D. Allen provided the Nomarski optics and made the movies. The illustrations are by Julia S. Child.

#### SUMMARY

1. Properties of *Botryllus schlosseri* which give it outstanding promise for studies in developmental genetics are reviewed.
2. Laboratory culture procedures, *in vitro* fertilization, and a method for raising embryos *in vitro* are described. Controlled successions of complete life cycles can now be achieved in any laboratory.
3. Experiments involving colony fusion, subsequent vascular budding, and the analysis of color patterns in resultant systems suggest that cells of the simple vessel walls govern the morphology of the regenerated zooids.
4. Results of some preliminary genetic crosses are reported.

#### LITERATURE CITED

- BANCROFT, F. W., 1903. Variation and fusion in compound ascidians. *Proc. Calif. Acad. Sci.*, series 3, 3: 137-186.
- BERRILL, N. J., 1941a. The development of the bud in *Botryllus*. *Biol. Bull.*, 80: 169-184.
- BERRILL, N. J., 1941b. Size and morphogenesis in the bud of *Botryllus*. *Biol. Bull.*, 80: 185-193.
- BERRILL, N. J., 1950. The Tunicata. Ray Society, London.
- BERRILL, N. J., 1951. Regeneration and budding in tunicates. *Biol. Rev.*, 27: 456-475.
- BERRILL, N. J., 1961. Growth, Development, and Pattern. Freeman, San Francisco.

- COLOMBERA, D., 1963. I cromosomi di *Botryllus schlosseri* (Ascidacea). *Ric. Sci.*, **33** (II-b), 443-448.
- COSTELLO, D. P., M. E. DAVIDSON, A. EGGERS, M. H. FOX AND C. HENLEY, 1957. Methods for Obtaining and Handling Marine Eggs and Embryos. Marine Biological Laboratory, Woods Hole, Mass.
- FREEMAN, G., 1964. The role of the blood cells in the process of asexual reproduction in the tunicate *Perophora viridis*. *J. Exp. Zool.*, **156**: 157-183.
- GROEBSTEIN, C., AND E. ZWILLING, 1953. Modification of growth and differentiation of chorio-allantoic grafts of chick blastoderm pieces after cultivation at a glass clot interface. *J. Exp. Zool.*, **122**: 259-284.
- GUILLARD, R. R. L., AND J. H. RYTHER, 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. *Canad. J. Microbiol.*, **8**: 229-239.
- LOPASCHOV, G., 1935. Die Entwicklungsleistungen des Gastrulamesoderms. *Biol. Zentralbl.*, **55**: 606-615.
- MILKMAN, R., AND M. BORGMANN, 1963. External fertilization of *Botryllus schlosseri* eggs. *Biol. Bull.*, **125**: 383.
- MILKMAN, R., AND S. BYRNE, 1961. Recent observations on *Botryllus schlosseri*. *Biol. Bull.*, **121**: 376.
- MILKMAN, R., AND E. THERRIEN, 1965. Developmental and genetic studies on the compound ascidian, *Botryllus schlosseri*. *Biol. Bull.*, **129**: 417.
- OKA, H., AND H. WATANABE, 1957. Vascular budding, a new type of budding in *Botryllus*. *Biol. Bull.*, **112**: 225-240.
- OKA, H., AND H. WATANABE, 1959. Vascular budding in *Botrylloides*. *Biol. Bull.*, **117**: 340-346.
- OKA, H., AND H. WATANABE, 1960. Problems of colony-specificity in compound ascidians. *Bull. Marine Biol. Sta. Asamushi*, **10**: 153-155.
- SABADDIN, A., 1958. Analisi sperimentale dello sviluppo delle colonie di *Botryllus schlosseri*. *Arch. Ital. Anat. Embriol.*, **63**: 178-221.
- SABADDIN, A., 1959. Analisi genetica del policromatismo di *Botryllus schlosseri* (Pallas) Savigny (Ascidacea). *Boll. Zool.*, **26**: 221-243. (Translation by Roger Milkman available on request.)
- SABADDIN, A., 1960. Ulteriori notizie sull'allevamento e sulla biologia dei Botrilli in condizioni di laboratorio. *Arch. Oceanogr. Limnol.*, **12**: 97-107.
- SABADDIN, A., 1962. Bande intersifonali di pigmento purinico in *Botryllus schlosseri* (Ascidacea) e loro determinazione genetica. *Boll. Zool.*, **29L**: 721-726.
- SABADDIN, A., 1964. The pigments of *Botryllus schlosseri* and their genetic control. *Ric. Sci.*, **34**: (II-b): 439-444.
- SCOTT, F. M., 1934. Studies on the later embryonic development of Tunicata: *Botryllus schlosseri* and *Amaroccium constellatum*. Ph.D. thesis, Columbia University, New York.
- VAN NAME, W. G., 1945. The North and South American ascidians. *Bull. Am. Mus. Nat. Hist.*, **84**: 1-476.
- WALTERS, D. R., AND C. M. WILLIAMS, 1966. Reaggregation of insect cells as studied by a new method of tissue and organ culture. *Science*, **154**: 516-517.
- WATKINS, M. J., 1958. Regeneration of buds in *Botryllus*. *Biol. Bull.*, **115**: 147-152.
- WATTERSON, R. L., 1945. Asexual reproduction in the colonial tunicate, *Botryllus schlosseri* (Pallas) Savigny, with special reference to the developmental history of intersiphonal bands of pigment cells. *Biol. Bull.*, **88**: 71-103.